Virology 278, 541–550 (2000)
doi:10.1006/viro.2000.0636, available online at http://www.idealibrary.com on IDEAL®

Expression and Folding of Human Very-Low-Density Lipoprotein Receptor Fragments: Neutralization Capacity toward Human Rhinovirus HRV2

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Received July 13, 2000; returned to author for revision August 30, 2000; accepted September 11, 2000; published online November 22, 2000

Minor group human rhinoviruses (HRVs) use members of the low-density lipoprotein receptor family for cell entry. To investigate the utility of receptor fragments as viral inhibitors, various polypeptide segments derived from the ligand binding domain of human very-low-density lipoprotein receptor (VLDLR) were expressed in a soluble form in bacteria. Whereas none of the fragments was active in virus binding immediately after recovery from the cell lysates, constructs encompassing complement type repeats 1-3, 1-6, and 1-8 spontaneously acquired virus binding activity by incubation at 4°C in buffer containing Ca^{2+} ions and lacking any redox system. When immobilized receptor-associated protein (RAP), a specific chaperone for VLDLR, was present during the incubation, the yield of protein active in ligand binding was substantially increased. A VLDLR fragment with repeats 4–6 failed to bind virus; however, it bound RAP. Bacterial expression of truncated VLDLR 1-3 at high yield, easy purification, and folding together with high inhibitory activity toward HRV2 makes this protein a promising starting point for the development of an oligopeptide-based antiviral agent. Using sucrose density gradient centrifugation, we demonstrate the formation of virus–receptor complexes. The recombinant receptors can thus be used for structure determination by electron cryo-microscopy.

INTRODUCTION

About 50% of all common cold infections are caused by human rhinoviruses (HRVs), members of the picornavirus family (Gwaltney, 1975). These are small (about 30 nm in diameter) icosahedral particles composed of 60 copies each of four capsid proteins constituting the viral shell. The genome is a single-stranded RNA with positive (messenger sense) polarity of about 7200 nucleotides in length (Rueckert, 1996). The family *Rhinoviridae* is divided into two groups. Major group viruses (91 serotypes) attach to human intercellular adhesion molecule 1 (ICAM-1) (Greve *et al.,* 1989; Staunton *et al.,* 1989; Tomassini *et al.,* 1989) and minor group viruses (10 serotypes) bind to low-density lipoprotein receptor (LDLR), to LDLR related protein (LRP), and to very-low density lipoprotein receptor (VLDLR) (Hofer *et al.,* 1994; Marlovits *et al.,* 1998b). HRV87 uses another glycosylated membrane protein of unknown function for cell entry (Uncapher *et al.,* 1991). Based on the susceptibility toward a panel of antiviral substances and the available sequence information, the International Committee for the Taxonomy of Viruses has recently divided the rhinoviruses into genus A and genus B (Andries *et al.,* 1990; King *et al.,* 2000). Drug binding appears to be strongly related to the shape of a hydrophobic pocket in the viral capsid, which determines how well the compounds are being accommodated (Andries *et al.,* 1991); members of one genus have a similar pocket geometry.

Soluble recombinant ICAM-1 has been shown to inhibit major receptor group rhinovirus infection by competition with cellular receptors and by inducing viral capsid modifications resulting in subviral particles sedimenting at 135S or 80S. The former lack the innermost capsid protein VP4, whereas the latter particles, which are considered the remainders of the uncoating process, in addition lack the genomic RNA (Casasnovas and Springer, 1994; Greve *et al.,* 1991; Hooverlitty and Greve, 1993; Korant *et al.,* 1972). Recombinant ICAM-1 without the transmembrane region and the cytoplasmic C-terminus as well as chimeric ICAM-1 molecules composed of immunoglobulin constant regions fused to the N-terminal two domains of human ICAM-1 have been shown to be potent viral inhibitors both *in vitro* (Martin *et al.,* 1993) and *in vivo* as demonstrated by clinical trials and animal experiments (Huguenel *et al.,* 1997; Turner *et al.,* 1999). Although about 90% of the HRV serotypes might respond to ICAM-1 treatment, all minor receptor group rhinoviruses belong to antiviral group B (genus rhinovirus B), whose members cause disease more often than members of group A (Andries *et al.,* 1990). It thus appears necessary to develop compounds active

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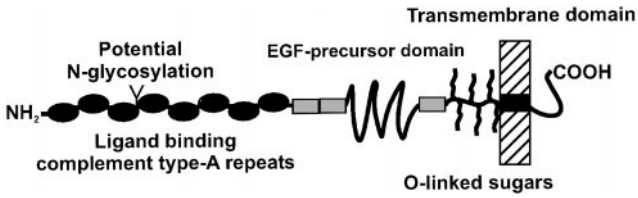

FIG. 1. Schematic drawing of the structure of the very-low-density lipoprotein receptor.

against minor group viruses in order to extend and complement a possible ICAM-1 medication.

The ligand binding domains of members of the LDLR gene family are all composed of different numbers of incomplete direct complement type A repeats (DiScipio *et al.,* 1984) of about 40 amino acids in length, each containing six cysteines that are all involved in disulfide bonds. LDLR has 7 such repeats (Yamamoto *et al.,* 1984), and VLDLR has 8 repeats (Takahashi *et al.,* 1992; see Fig. 1 for a schematic representation of this molecule) and LRP has clusters of 2, 8, 10, and 11 (Herz *et al.,* 1988) repeats (for a review on the LDLR gene family see, e.g., Gliemann (1998); Strickland *et al.* (1995)). The ligand binding domain is followed by a region with similarity to epidermal growth factor precursor, a highly O-glycosylated domain, the transmembrane domain, and a C-terminal intracellular region that contains internalization signals. Using eukaryotic expression in Sf9 insect cells of LDL minireceptors encompassing less than the seven complement type A repeats, we recently determined the minimal structure requirements for virus recognition and cell protection. LDLR fragments with more than two repeats were able to protect HeLa cells against infection with the minor group rhinovirus HRV2, whereas two repeats were sufficient for recognition of the virus in a ligand blot-type assay (Marlovits *et al.,* 1998a). Apparently, the affinity of the smallest minireceptors was insufficient for competition with the cellular receptors when free in solution but increasing the avidity by immobilization on PVDF membranes allowed for attachment of the virus over multiple sites. In contrast to ICAM-1, LDL receptors do not uncoat minor group HRVs *in vitro* and their cell protection activity thus appears to rely solely on competition with the cellular receptors (Gruenberger *et al.,* 1995) or on aggregation (Marlovits *et al.,* 1998c).

Earlier experiments with the detergent-solubilized chicken homologue of mammalian VLDLR suggested that this receptor might bind virus with higher affinity than LDLR (Gruenberger *et al.,* 1995); complexes between virus and this receptor could be isolated on sucrose density gradients, whereas this was not possible with soluble recombinant LDLR. The latter recombinant protein with seven ligand binding repeats was shown rather to induce aggregation most probably via oligomeric forms present in the preparation (Marlovits *et al.,* 1998c). With the aim of engineering a small receptor derivative efficient in cell protection and thus suitable as a starting point for the development of an antivirally active oligopeptide we set out to express soluble fragments derived from human VLDLR. As already seen for soluble LDL minireceptors (Marlovits *et al.,* 1998a), expression in the baculovirus system revealed that the entire ligand binding domain of VLDLR could be easily purified making use of a hexa-his tag appended at its C-terminus. Nevertheless, upon subsequent enrichment of the correctly folded form by affinity chromatography on columns containing the common ligand of all members of the LDLR gene family, receptor-associated protein (RAP) (Medh *et al.,* 1995), we discovered that only small amounts of the total protein were active in ligand binding (Marlovits *et al.,* 1998b).

Recently, it was shown that single LDLR repeats (Bieri *et al.,* 1995; Daly *et al.,* 1995a, b), a two-repeat oligopeptide (Bieri *et al.,* 1998), a fragment encompassing three repeats (C5-C7) from LRP (Vash *et al.,* 1998), and even the entire ligand binding domain of LDLR (Simmons *et al.,* 1997) could be expressed in bacteria and refolded from the completely denatured form to native structures using a complicated protocol. This prompted us to attempt bacterial expression and folding of truncated human VLDLR. Employing the expression system that makes use of fusion of the foreign polypeptides with maltose binding protein (MBP) we discovered that the proteins folded spontaneously upon storage at 4°C without the need for complex denaturing/renaturing protocols. Furthermore, the yield of active soluble VLDLR minireceptors was greatly improved when folding was carried out in the presence of Sepharose-immobilized glutathione *S*-transferase RAP (GST–RAP). We here report on the prokaryotic expression, purification, and folding of various recombinant VLDLR fragments and demonstrate their antiviral activity toward HRV2 *in vitro* by protection of HeLa cells against viral infection. In addition we show that complexes between virus and recombinant receptor fragments containing the ligand binding domain only are stable enough to be purified on sucrose density gradients. This opens the way for the determination of the three-dimensional structure of these complexes and will allow the identification of the receptor binding site by electron microscopy techniques.

RESULTS

Expression of soluble human VLDLR fragments

The cDNAs encoding VLDLR fragments containing repeats 1-3, 4-6, 1-6, and 1-8 were excised from the respective pTM1 clones together with the sequence encoding his_6 and inserted into pMalc2b (see Table 1). Following induction and lysis of bacteria harboring the plasmids, high-speed supernatants were prepared and analyzed in parallel with the pellets for the presence of the recombinant proteins by sodium dodecyl sulfate-

TABLE 1

Name of primer	Sequence (5′ → 3′)[a]	Location[b]
TIM371	**ATGC**GGATCCAGGGAGAAAAGCCAAATGTG[c]	82–100, upstream of repeat 1
TIM522	**GCAG**CCCGGGATTGCCACAGTTTTCTTCATCTTCTCC	497–523, downstream of repeat 3
TIM435	**CTATGC**GGATCCAATAACATGTAGTCCCGACGAGTTCACC	524–550, upstream of repeat 4
TIM436	**GCAG**CCCGGGAGAGGGACAGTTGACCTCATCACTGCC	868–895, downstream of repeat 6
TIM372	**GCTG**CCCGGGACACTCTTTCAGGGGCTCAT[c]	1046–1066, downstream of repeat 8

[a] Nucleotides shown in boldface type are not complementary to the VLDLR cDNA but were added to create restriction sites for *Bam*HI or *Xma*I (underlined).

[b] Numbers given correspond to the position of complementarity to human VLDLR cDNA. For orientation, the repeat number is also indicated.

[c] See also Marlovits *et al.* (1998b).

polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. This revealed that more than 90% of the proteins were present in the soluble fraction (data not shown). The receptor fragments were thus purified from the supernatants on Ni-NTA (Ni^{2+} nitrilotriacetic acid) resin without requiring solubilization. Aliquots corresponding to 0.1 ml of bacterial culture were then analyzed by SDS–PAGE under reducing conditions followed by Coomassie brilliant blue staining. As seen in Fig. 2, all proteins were expressed with similar yields and showed apparent M_rs somewhat higher than those calculated from the amino acid sequence (Table 2).

To test for the presence of correctly folded proteins, SDS–PAGE was repeated under nonreducing conditions followed by electrophoretic transfer of the separated proteins to a PVDF membrane. The membrane was then incubated with ^{35}S-labeled HRV2 and exposed to X-ray film. In contrast to $VLDLR_{1-8}$ and other receptor fragments expressed in Sf9 insect cells (Marlovits *et al.,* 1998b; and unpublished results), all bacterially produced proteins lacked ligand binding activity (data not shown). However, when the samples were kept at 4°C and the analysis was repeated 3 days later, all VLDLR fusion proteins, except MBP-$VLDLR_{4-6}$, turned out to bind HRV2 (Fig. 3A). As all recombinant receptor fragments were found to bind GST–RAP, the two ligands appear to have different structure requirements for recognition (Fig. 3B). Coomassie staining of a gel run in parallel with identical samples showed that all proteins were present at comparable amounts (Fig. 3C). In some experiments, dimers, which also bound both virus and RAP, were observed in small amounts (see, e.g., first lane in Fig. 3A). Dimers arising from intermolecular disulfide bonds have been observed previously for LDLR fragments expressed in insect cells (Marlovits *et al.,* 1998c). Using MBP-$VLDLR_{1-6}$ as an example, the time course of the folding reaction was then investigated.

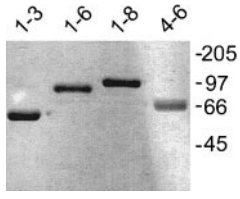

FIG. 2. Bacterial expression of human VLDLR fragments fused to maltose-binding protein and carrying a hexa-his tag at their C-termini. *E. coli* TB1 harboring the respective plasmids were induced with IPTG and bacterial lysates were prepared. Recombinant proteins from 0.1 ml of bacterial culture were purified by Ni-NTA metal chelate chromatography and separated under reducing conditions on a 12% polyacrylamide gel in the presence of SDS. Proteins were stained with Coomassie brilliant blue. Complement type A repeats contained in the respective proteins are indicated at the top of the lanes. Apparent relative molecular mass (kDa) of marker proteins run on the same gel is also shown.

Spontaneous folding of human $VLDLR_{1-6}$

Immediately after elution from the Ni-NTA column, an aliquot of MBP-$VLDLR_{1-6}$ corresponding to 0.1 ml of bacterial suspension was frozen at −20°C. The remainder was then left at 4°C, and at the times indicated in Fig. 4 samples were taken and kept frozen at −20°C until analysis by PAGE under nonreducing conditions followed by transfer to PVDF membranes and ligand blotting with radiolabeled HRV2. As seen on the autoradiograph (Fig. 4), virus binding activity was absent for up to

TABLE 2

Name of recombinant protein[a]	From AA No. to AA No. of human VLDLR	Calculated M_r[b]
MBP-$VLDLR_{1-3}$	28–151	59,000
MBP-$VLDLR_{1-6}$	28–274	72,000
MBP-$VLDLR_{1-8}$	28–355	81,000
MBP-$VLDLR_{4-6}$	152–274	59,000
Truncated MBP[c]		43,000

[a] Subscripts refer to the repeats contained.

[b] Including the oligopeptide sequence ISEFADP N-terminal of the VLDLR fragment and the peptide sequence PGLQSRPGGPGQGSGGHHHHHH C-terminal of the VLDLR fragment, but excluding the leader peptide; calculations also include MBP and the cleavage site for factor Xa.

[c] The MBP encoded in the vector is truncated at the C-terminus and contains amino acids encoded in the multiple cloning site.

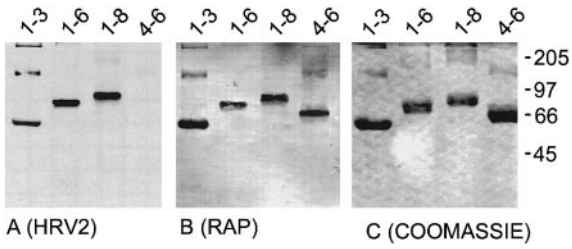

FIG. 3. After spontaneous folding, MBP-VLDLRs bind ^{35}S-labeled HRV2 (A) and biotinylated GST–RAP (B) in ligand blots. Following elution of the recombinant proteins from Ni-NTA columns they were allowed to fold by incubation at 4°C for 3 days. Samples corresponding to 0.1 ml of bacterial culture were applied to SDS 12% polyacrylamide gels, separated under nonreducing conditions, and transferred to PVDF membranes for ligand blotting. To monitor the total amount of protein, an identical gel was also stained with Coomassie brilliant blue (C). Positions and corresponding relative molecular mass (in kDa) of marker proteins are also shown.

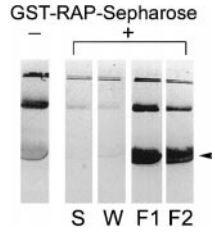

FIG. 5. RAP-assisted folding of MBP-VLDLR$_{1\text{-}3}$ fusion protein. MBP-VLDLR$_{1\text{-}3}$ was purified from bacterial lysates on a Ni-NTA column. Three hundred microliters of GST–RAP–Sepharose was added to 1 ml (0.8 mg) of the eluate and the sample was incubated at 4°C under gentle mixing for 3 days. The resin was pelleted and the supernatant (S) was collected. After being washed with 500 μl of 0.5X TBS-Ca (W), bound protein was eluted with 2X 100 μl of 1 M NH_3 in 0.5X TBS-Ca (F1 and F2). Aliquots corresponding to 1/20 of the original volume were analyzed for ligand binding by separation of the proteins on SDS 12% polyacrylamide gels run under nonreducing conditions. The proteins were transferred to PVDF membranes and virus binding activity was visualized by incubation with ^{35}S-labeled HRV2 followed by exposure to X-ray film for 1 day. For comparison, the original sample was incubated in the absence of GST–RAP–Sepharose (–). The monomeric form of MBP-VLDLR$_{1\text{-}3}$ is indicated with an arrowhead.

1 h after elution from the column, but appeared at about 6 h and continually increased with time. Moreover, at early time points virus bound preferentially to high-molecular-weight aggregates of the receptor protein but the amount and virus binding activity of the monomeric form increased over the time of incubation.

Folding of recombinant MBP-VLDLR fragments assisted by immobilized GST–RAP

RAP has been shown to act as a molecular chaperone for members of the LDLR family presumably by preventing aggregation of the receptors with their ligands in the endoplasmic reticulum (Bu *et al.,* 1995; Willnow *et al.,* 1995). In addition, together with Ca^{2+} ions, it is required for proper folding of LRP (Obermoeller *et al.,* 1998) and of VLDLR (Sato *et al.,* 1999). We thus argued that folding might be promoted in the presence of RAP. Therefore, Ni-NTA purified MBP-VLDR fragments were incubated with GST–RAP Sepharose at 4°C for 3 days under gentle agitation. As correctly folded protein was expected to remain bound to the resin, it was then poured into a column, washed with 0.5X TBS-Ca and bound protein was eluted with 1 M NH_3 in 0.5X TBS-Ca. Ammonia was removed and the volume was reduced by a factor of 2 in a Speedvac concentrator. In order to estimate the fraction of correctly folded material contained in the total protein expressed, aliquots from flowthrough, wash, and eluate were analyzed by PAGE followed by ligand blotting with radiolabeled HRV2. As exemplified for MBP-VLDLR$_{1\text{-}3}$ on the autoradiograph depicted in Fig. 5, the fraction of material active in virus binding was substantially increased by this procedure. Determination of the radioactive virus bound to the monomeric form of the receptor (see arrowhead) with an Instant Imager revealed that the combined eluates (fractions 1 and 2 in Fig. 5) exhibited a four times higher binding activity toward HRV2 than the corresponding material that had been incubated in the absence of GST–RAP Sepharose (leftmost lane in Fig. 5).

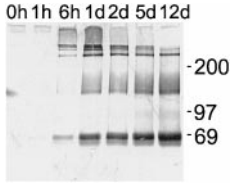

FIG. 4. Time course of folding of MBP-VLDLR$_{1\text{-}6}$ fusion protein. MBP-VLDLR$_{1\text{-}6}$ was purified from bacterial lysates on a Ni-NTA column. Immediately after elution, an aliquot was frozen at −20°C and the remainder was kept at 4°C. At the times indicated at the top of the lanes, samples were taken and kept frozen until analysis by SDS–PAGE in 10% gels under nonreducing conditions followed by ligand blotting with radiolabeled HRV2. Samples correspond to 0.1 ml of bacterial culture. The positions of ^{14}C-labeled marker proteins (given in kDa) separated on the same gel and transferred to the ligand blot are also indicated.

Virus-neutralization activity of the recombinant VLDLR fragments

Having demonstrated that VLDLR fragments could be easily expressed in bacteria and spontaneously folded without the need for tedious denaturation/refolding protocols we determined the cell protection activity of the recombinant fusion proteins toward the minor group rhinovirus HRV2 following the procedure previously used for LDL minireceptors (Marlovits *et al.,* 1998a). Briefly, 100 $TCID_{50}$ of HRV2 was incubated with twofold serial dilutions of the Ni-NTA purified receptors that had been folded in the presence of GST–RAP as pointed out above, and the mixtures were transferred onto HeLa cell monolayers grown in 96-well microtiter plates. After 5 days of

TABLE 3

Recombinant soluble receptor[a]	Number of repeats	MIC_{50} (nmol/liter)[b]
$rLDLR_{3-5}h$	3	180
$rLDLR_{3-7}h$	5	12
$rLDLR_{1-5}h$	5	69
$rLDLR_{1-7}h$	7	62
$MBP\text{-}VLDLR_{1-3}$	3	79
$MBP\text{-}VLDLR_{4-6}$	3	Inactive
$MBP\text{-}VLDLR_{1-6}$	6	16
$MBP\text{-}VLDLR_{1-8}$	8	n.d.[c]

[a] Values for recombinant LDLR fragments were taken from Marlovits *et al.* (1998a) and converted to nmol/liter.

[b] Values were obtained from a cell protection assay by determination of A_{560} after the stain was dissolved in 100 μl of 1 M NaOH as described previously (Marlovits *et al.,* 1998c).

[c] Not determined.

incubation at 34°C, cells remaining bound to the plastic were stained with Amido black. The stain was then dissolved in NaOH and quantified with a microplate reader (Marlovits *et al.,* 1998c). The minimal inhibitory concentrations, affording 50% protection of the cell monolayer (MIC_{50} (Arruda *et al.,* 1992)), were compared with those of LDL minireceptors (Marlovits *et al.,* 1998a). From this it becomes clear that $rLDLR_{3-7}h$ was most active against HRV2 closely followed by $MBP\text{-}VLDLR_{1-6}$. From the smallest minireceptors, $MBP\text{-}VLDLR_{1-3}$ was found to be more active than $rLDLR_{3-5}h$ (Table 3). Similar results were also obtained with another minor group virus, HRV1A; the major group virus HRV14, used as a control, was not inhibited (data not shown). Due to the large number of possible combinations of the repeats present in LDLR and VLDLR it is not possible to rule out the possibility that another combination of the repeats present in these receptors would be even more active in cell protection against minor group virus infection. Whether the cell protective activity is directly related to the affinity of the receptor derivatives for the virus is currently being investigated using plasmon surface resonance technology.

Formation of complexes between virus and receptor

Various attempts to isolate complexes between HRV2 and recombinant LDL receptor fragments have failed, most probably due to extensive aggregation (Marlovits *et al.,* 1998c) or too low affinity (Marlovits *et al.,* 1998a). Although virus/receptor complexes formed between HRV2 and detergent solubilized OVR (Gruenberger *et al.,* 1995) they could not be visualized by electron microscopy (unpublished results). We therefore asked whether recombinant human VLDL receptors were able to associate with the virus in solution and assemble complexes stable enough to be separated from excess receptor by sucrose density gradient centrifugation. Assuming 60 receptor binding sites, HRV2 was incubated with a threefold molar excess of $MBP\text{-}VLDLR_{1-8}$ and the mixture was applied onto sucrose gradients formed in 800-μl centrifuge tubes. After ultracentrifugation, tubes were pierced at the bottom and fractions corresponding to two drops each were collected. Proteins present in the fractions were then determined by polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. As seen from the presence of the viral capsid proteins VP1–VP3, virus alone sedimented to the lower third of the gradient under these conditions (Fig. 6A). When the mixture of virus and receptor was analyzed under identical conditions, the receptor was found to comigrate with the virus at a position somewhat shifted toward the lower density of the gradient (Fig. 6B). This indicates that the sedimentation coefficient of the complex is slightly less than that of the virus alone, which sediments at 150S (Korant *et al.,* 1972). Finally, receptor alone does not enter the gradient at all in the absence of virus, which indicates the absence of aggregates and excludes accidental comigration (Fig. 6C).

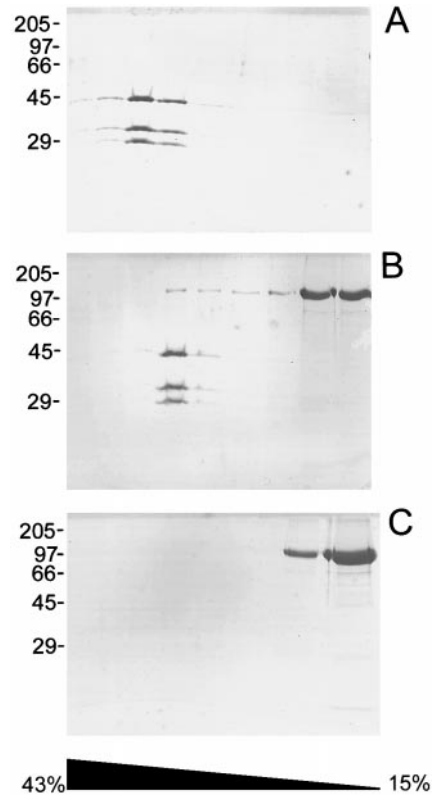

FIG. 6. Sucrose density gradient centrifugation reveals the formation of complexes between HRV2 and $MBP\text{-}VLDLR_{1-8}$. Virus was incubated with receptor for 1 h at room temperature and applied onto sucrose density gradients formed in 800-μl centrifuge tubes. After centrifugation, fractions collected from the bottom (left) were analyzed by SDS–12% polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. (A) Virus incubated without receptor; (B) virus incubated with receptor; (C) receptor without virus. Positions of marker proteins run on the same gels are indicated on the right. Note that the gradients had a sigmoid shape as determined with a refractometer.

DISCUSSION

We have shown earlier that LDL minireceptors with cell protective activity against minor group HRV infection could be expressed in the baculovirus system (Marlovits *et al.,* 1998a). However, although the amount of total protein was rather high, we observed that only a small fraction was present in a correctly folded state, requiring ligand affinity chromatography as an additional means of enriching for the protein active in ligand recognition (Marlovits *et al.,* 1998a). For this purpose, a ligand of LDLR, rabbit β-VLDL, was used. However, production of this lipoprotein is not as reproducible as that of recombinant GST–RAP, which has low affinity toward LDLR but very high affinity toward VLDLR (Medh *et al.,* 1995). GST–RAP Sepharose was thus used for purification of a recombinant soluble VLDLR fragment encompassing the entire ligand binding domain (repeats 1–8) expressed in Sf9 insect cells. This fragment was shown to be active in protection of HeLa cells against infection with HRV2 (Marlovits *et al.,* 1998b). In order to investigate the activity of smaller VLDLR fragments with respect to cell protection against minor group rhinovirus infection, expression of soluble VLDLRs in bacteria was attempted. Whereas LDLR contains three potential glycosylation sites, VLDLR has only one; this site is located exactly at the border between repeat 3 and repeat 4 and is thus not contained in the receptor fragments $VLDLR_{1\text{-}3}$ and $VLDLR_{4\text{-}6}$. Therefore, we argued that expression in bacteria would more easily result in active proteins than in the case of the LDLR fragments where glycosylation might be required for correct folding. We thus investigated the utility of the pMal system for bacterial expression of the desired protein as a fusion to maltose binding protein. The commercial plasmid pMalc2x was modified by introduction of a novel multiple cloning site and a hexa-histidine tag that becomes appended at the C-terminus of the recombinant protein upon expression under the control of the strong tac promoter. However, all recombinant VLDL receptor fragments turned out to be inactive in virus binding when tested immediately after elution from the Ni-NTA column but acquired virus binding activity upon storage at 4°C within 1 to 2 days. This occurred concomitantly with the rearrangement of disulfide bonds as high-molecular-weight oligomers gradually disappeared, giving rise to monomeric forms of the proteins. We have not investigated folding of the VLDLR fragments in the absence of MBP. Therefore, the possibility cannot be excluded that this particular fusion partner takes a major role in folding or in rendering the fragments more soluble. Recently, Kapust and colleagues compared the solubility of foreign proteins upon fusion to MBP, thioredoxin, and glutathione *S*-transferase, respectively, upon expression in *Escherichia coli* and found the former to be most effective (Kapust and Waugh, 1999).

As RAP was shown to act as a chaperone for members of the LDLR gene family (reviewed by Bu and Schwartz (1998)) we argued that its presence would promote the folding process. Incubation of the column eluates with Sepharose-immobilized GST–RAP indeed resulted in a substantial increase in virus binding activity and at the same time allowed for additional purification as only correctly folded material remained bound to the column material and was subsequently eluted. So far, the smallest VLDLR fragments investigated are $VLDLR_{1\text{-}3}$ and $VLDLR_{4\text{-}6}$; whereas the former was found to be active in a cell protection assay, the latter was not.

Our results are at variance with those of a recent report by Savonen *et al.* (1999), who presented evidence for the binding site of RAP residing in the first three repeats of VLDLR. We found that both MBP-$VLDLR_{1\text{-}3}$ and MBP-$VLDLR_{4\text{-}6}$ were retained on the GST–RAP column and that both bound biotinylated GST–RAP on ligand blots as well. This clearly demonstrates the presence of more than one RAP binding site on the native receptor.

Comparison of MBP-$VLDLR_{1\text{-}3}$ with the LDL minireceptor $rLDLR_{3\text{-}5}h$ revealed a higher cell protection activity of the former protein. Whereas the N-termini of the recombinant LDL minireceptors were identical to that of the native receptor, the bacterially expressed VLDLR fragments investigated were N-terminally fused to MBP. It is thus interesting to note that this fusion had no major influence on virus binding as seen upon enzymatic removal of MBP with factor Xa (data not shown). This leaves us with the question of how the interaction between virus and receptor takes place. In major group viruses, the N-terminal immunoglobulin-like domain of ICAM-1 binds within the canyon, a deep cleft encircling the fivefold axes of icosahedral symmetry of the viral capsid (Olson *et al.,* 1993). It thus appears unlikely that receptors carrying MBP as an N-terminal extension of 43 kDa can be accommodated within an equivalent position. Based on the above findings it appears probable that the receptors attach to sites more exposed than the interior of the canyon. Nevertheless, a more inclined position, similar to that of the poliovirus receptor, whose interactions with the virus extend beyond the canyon (Belnap *et al.,* 2000; He *et al.,* 2000; Xing *et al.,* 2000), might permit lateral contacts, keeping the N-terminally fused MBP free to stick out in solution.

The number of repeats present in LDLR and VLDLR and their difference in sequence preclude an analysis of all imaginable combinations. It is thus possible that receptor fragments with three or even two repeats exhibit higher cell protective activity. The comparison between $VLDLR_{1\text{-}3}$ and $VLDLR_{4\text{-}6}$ suggests, however, that particular combinations of the repeats determine viral binding affinity. Nevertheless, the possibility cannot be excluded that artificial combinations, such as oligomers assembled from the same repeat, can act as potent viral inhibitors as well. In conclusion, the simplicity of expression, folding, and purification of MBP-$VLDLR_{1\text{-}3}$ is highly sug-

gestive for its use as a starting point for the development of an antivirally active drug as preliminary data indicate that the maltose binding protein moiety can easily be removed by cleavage with factor Xa without loss of cell protection activity. Experiments aimed at increasing the affinity of this receptor fragment for minor group HRVs by mutagenesis and selection will be carried out in the future.

Many attempts to isolate stable complexes between HRV2 and various recombinant LDLR fragments have been unsuccessful. We think that the affinity of LDLR or of the fragments thereof is too low for the components to remain associated during the separation process such as sucrose density gradient centrifugation or spin column separation (Hewat and Blaas, 1996). Nevertheless, the receptor fragments proved very active in protecting the cells against viral infection (Table 3). Similar observations have also been made with recombinant decay accelerating factor and echovirus 7 (Powell *et al.,* 1997). MBP-VLDLR$_{1-8}$ (Fig. 6) and VLDLR$_{1-3}$ (to be published elsewhere) have now been shown to attach sufficiently strongly to HRV2 to allow for the isolation of complexes. Analysis of these complexes by electron cryo-microscopy followed by image reconstruction is now under way.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma, unless specified otherwise. Isopropylthio-β-D-galactoside (IPTG) and fetal calf serum were obtained from Life Technologies. *Pfu* polymerase was from Stratagene; restriction enzymes, T4-DNA ligase, pMalc2x, and bacterial strain TB1 were from New England Biolabs. Ni-NTA beads were from Qiagen. GST–RAP was prepared as described (Herz *et al.,* 1991) and covalently linked to CNBr-activated Sepharose following the manufacturer's protocol (Amersham Pharmacia Biotech). About 4.5 mg of GST–RAP per milliliter of settled Sepharose beads was bound as determined by the modified Lowry procedure (Cadman *et al.,* 1979). HRVs were from the American Type Culture Collection. Rhino HeLa cells (Flow Laboratories) were used throughout. HRV2 was metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine (ARS110, American Radiolabeled Chemicals) as described (Neubauer *et al.,* 1987). ^{14}C-labeled marker proteins were from Amersham Life Sciences. Polyvinylidene difluoride (PVDF) membranes were from Millipore. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin) and alkaline phosphatase conjugated streptavidin were purchased from Pierce. Nitro-blue tetrazolium chloride (NBT) was from Amresco and dissolved in 70% dimethyl formamide at 50 mg/ml. 5-Bromo-4-chloro-3-indolyl-phosphate, disodium salt (BCIP) was from Calbiochem and dissolved at 50 mg/ml in water.

Cloning and expression of soluble VLDLR fragments

Following the protocol described previously for the construction of recombinant baculovirus vVLDLR$_{1-8}$h containing the entire ligand binding domain (Marlovits *et al.,* 1998b), combinations of the synthetic oligonucleotide primers listed in Table 1 were used for PCR amplification of cDNA fragments encompassing complement type A repeats 1-3, 4-6, and 1-6 from pVLDLR-1 (Sakai *et al.,* 1994). For completeness, primers used previously for amplification of repeats 1–8 are also included in Table 1. After digestion with *Bam*HI and *Xma*I, the DNAs were purified by agarose gel electrophoresis and adsorption to glass milk; eluted DNA was then ligated into the transposition vector pTM1 to also allow for use of the baculovirus expression system (Marlovits *et al.,* 1998a). For bacterial expression of VLDLR fragments with MBP fused to their N-termini, pMalc2x was modified by removing the multiple cloning site by digestion with *Eco*RI and *Hin*dIII and introduction of a new polylinker sequence resulting from ligation with a double-stranded linker DNA obtained by annealing synthetic oligonucleotides with the sequences 5′-AATTCGCGGATCCCCATGGGGTACCA-3′ and 5′-AGCTTGGTACCCCATGGGGATCCGCG-3′. The resulting vector was termed pMalc2b and used for all following constructions. The cleavage site for factor Xa encoded in pMalc2x was conserved. The new vector thus created allowed for the direct introduction of *Bam*HI and *Kpn*I fragments excised from the respective TM1 constructs (see above) together with the hexa-his tag sequence (see Table 1).

E. coli TB1 were transformed with the respective plasmids and induced at a density corresponding to A_{600} of 0.8 with 0.4 mM IPTG at 30°C for 2 h. Cells were pelleted, suspended in 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ (TBS-Ca; 5 ml per 1 g of moist pellet) containing 1 mM phenylmethylsulfonyl fluoride, and broken by sonication. Insoluble material was removed by centrifugation at 18 krpm for 30 min using an SS34 fixed angle rotor in a SorvaII centrifuge, and the recombinant proteins corresponding to 5 ml of bacterial lysate were purified over a 2-ml Ni-NTA column. VLDLR fragments were termed MBP-VLDLR$_{n-m}$ with the subscripts referring to the repeats contained. The segments from native human VLDLR present in the recombinant proteins and their calculated relative molecular mass are summarized in Table 2.

Ligand blotting

Ligand binding activity of the purified fusion proteins was revealed with HRV2 metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine (Neubauer *et al.,* 1987) after SDS–PAGE under nonreducing conditions and electrophoretic transfer to PVDF membranes as described previously (Marlovits *et al.,* 1998c). Bound virus was visualized by autoradiography on Kodak Biomax film. RAP

binding to recombinant receptor molecules was monitored using biotinylated GST–RAP.

Recombinant GST–RAP was dialyzed against PBS, and NHC-LC-biotin, dissolved in DMSO, was added in a 100-fold molar excess. After incubation for 3 h at 4°C the reaction was quenched by addition of 1/10 vol of 1 M Tris–HCl (pH 9) and the material was dialyzed against TBS-Ca. To monitor RAP binding, the membrane was incubated with biotinylated GST–RAP at 1 μg/ml followed by a 1:10,000 dilution of streptavidin–AP conjugate. Bound streptavidin was detected with 66 μl NBT and 33 μl BCIP stock solution per 10 ml of AP buffer (100 mM Tris–HCl (pH 9.6), 100 mM NaCl, 5 mM $MgCl_2$). Blocking and washing of the membranes with 2 and 0.1% Tween 20, respectively, were carried out as described for HRV2 (Marlovits *et al.,* 1998c).

Folding of MBP-VLDLR$_{n-m}$

After elution from the Ni-NTA metal chelate column the samples were stored at 4°C without further treatment. At different time periods, aliquots were removed and frozen until all samples were subjected simultaneously to PAGE under nonreducing conditions. Proteins were transferred onto PVDF membranes and virus binding activity was revealed by incubation with ^{35}S-labeled HRV2. In parallel, an identical gel was stained with Coomassie brilliant blue. For RAP-assisted folding, 4 ml of the metal chelate column eluates (12 mg total protein) was added to 6 ml of settled GST–RAP Sepharose. The suspension was mixed by slowly rotating the tube end over end in the cold room. After 2 days the gel was transferred into a column, the liquid was drained, the column material was washed with 30 ml of 0.5X TBS-Ca, and bound protein was eluted with 4 ml of 1 M NH_3 in 0.5X TBS-Ca. NH_3 was removed and the samples were concentrated two times in a Speedvac concentrator.

Cell protection assays

Cell protection assays were carried out as described previously (Marlovits *et al.,* 1998c). Briefly, HRV2 at 100 $TCID_{50}$ was incubated in infection medium (MEM containing 2% heat-inactivated fetal calf serum and 30 mM $MgCl_2$) in a final volume of 100 μl with serial twofold dilutions of purified recombinant VLDLR fragments for 90 min at 34°C. HeLa cell monolayers in 96-well plates were then challenged with the mixtures and incubated for 5 days at 34°C. Media were removed and cells having remained attached to the plastic surface were stained with Amido black (0.1% in acetic acid, methanol, water; 10/40/50 vol/vol). For quantification, the stain was dissolved in 100 μl of 1 M NaOH and A_{560} was determined in a microplate reader (Marlovits *et al.,* 1998c).

Sucrose gradient centrifugation

To resolve MBP-VLDLR$_{1-8}$ from any oligomers present in the GST–RAP Sepharose column eluate the material was run in TBS-Ca over a Superdex 200 column connected to a Pharmacia FPLC system. The peak fraction corresponding to the monomer was concentrated five times in a Speed Vac concentrator and 60 μg MBP-VLDLR$_{1-8}$ (740 pmol) was incubated with 30 μg HRV2 (3.75 pmol) in a final volume of 50 μl for 1 h at room temperature and was layered onto sigmoid sucrose density gradients (45–15%) formed by diffusion in 800-μl cellulose nitrate tubes (Stone, 1974). Centrifugation was for 80 min at 35 krpm in a Beckman SW50.1 rotor equipped with suitable adapters. The tubes were connected to a peristaltic pump at their top and pierced at the bottom with a hypodermic needle, and two-drop fractions were collected by slowly pumping air into the tubes after removal of the needle. The fractions were analyzed by SDS–polyacrylamide gel electrophoresis on 12% minigels under reducing conditions followed by Coomassie brilliant blue staining.

ACKNOWLEDGMENTS

This work was funded by the Austrian Science Foundation Grant P12189-MOB. We thank I. Goesler for excellent tissue culture work and virus production.

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